Is the effect of Decitabine limited to hypomethylation and DNMTs?

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Research article

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Abstract

Background: Histone modifications play a crucial role in chromatin structure. Among enzymes, which regulate these processes, histone deacetylases (HDACs) can remove acetyl groups from histone tails, thus increasing their interaction with DNA and leading to chromatin condensation. 5-Aza-2'-deoxycytidine (AZad) or Decitabine is a potent hypomethylating agent that incorporates into DNA and traps DNA methyltransferase in the form of a covalent protein–DNA adduct. Azad, not only change the gene expression through demethylation of the gene's promoter, but it also can change gene expression independently from DNA demethylation. So, the present study was to distinguish whether AZad in addition to inhibitory effects on DNA methyltransferase, can change HDAC3 and HDAC7 mRNA expression in NALM-6, HL-60 cancer cell lines. Methods: HL-60, NALM-6 and normal cells were cultured, and the treatment dose of the AZad was obtained (1μM) by the MTT test. Finally, HDAC3 and HDAC7 mRNA expression were measured by Real Time PCR in HL-60 and NALM-6 cancerous cells before and after treatment. In addition, HDAC3 and HDAC7 mRNA expression in un-treated HL-60 and NALM-6 cancerous cells were compared to the normal cells. Results: Our result revealed that expression of HDAC3 and HDAC7, in HL-60 and NALM-6 cells increases as compared to normal cells. After treatment of HL-60 and NALM-6 cells with AZad, HDAC3 and HDAC7 mRNA expression were decreased significantly. Conclusions: Our data showed, the effects of AZad are not limited to direct hypomethylation of DNMTs but it can indirectly affect other epigenetic factors, such as HDACs activity, through converging pathways.

Keywords: HDAC3 ; HDAC7 ; HL-60; NALM-6 ; Decitabine ; AZad

Background

Epigenetics changes is the reason why, all body cells have the same DNA but with the different gene expression pattern, which leads to the formation of different cells types (1). These changes are inherited and have no effect on DNA nucleotide sequence (2). There are multiple types of epigenetic mechanisms such as the regulation of non-coding RNA, DNA methylation; by addition of methyl groups to a part of the DNA molecule. Histone modifications; by addition of acetyl group, methyl group, phosphors groups, ADP-ribosylation, ubiquitylation, etc. (3, 4). Histones modifications can result in alteration of gene transcription, this modifications carry out by relevant enzymes (5). Generally, histones are proteins that contribute to pack DNA through the creation of nucleosomes. There are five recognized type of histones including H1, H2A, H2B, H3, H4 and H5. The histones H2A, H2B, H3 along with H4 form the nucleosomes. Histone H5 exist in specific area of the DNA and histone H1 is observed in the places between nucleosomes (6, 7). The principal enzymes for monitoring of histone regulation are Histone methyltransferases (HMTs), Histone demethylases (HDMs), Histone acetyltransferase (HATs) and Histone deacetylases (HDACs) (5). HDACs are classified into 4 groups; group I HDACs, including HDAC 1, 2, 3 and 8, present in nucleus, group II HDACs, including HDAC 4, 5, 6, 7, 9 and 10, move between the nucleus and cytoplasm (8). Whereas, often class I HDACs are ubiquitously expressed, but the class IIa HDACs are expressed in a limited number of various cells (9). HDACs do not connect to DNA directly, and it is believed that to be recruited to special promoters via their interaction with DNA sequence-specific

transcription agents (10). Some studies confirmed that HDAC inhibitors (HDACi) can cause apoptosis and stop cell cycle in acute lymphoblastic leukemia (ALL) cell cultures (11, 12). Some studies show that, HDAC7 and HDAC9 mRNA overexpression was recognized to be associated with bad prognostic agents and a higher risk of unfavorable results (13). Moreover, it has been shown that HDAC7 may control the beginning of apoptosis (14). High expression of HDAC7 also observed in pancreas adenocarcinomas (15). Thus, subsequently epigenetic drugs were developed to treat various diseases. There are many epigenetic drugs such as, bromodomain and inhibitors, HAT inhibitors, protein methyltransferase inhibitors, histone methylation inhibitors, methylation inhibiting and HDAC inhibitors drugs. 5-Aza-2'-deoxycytidine (Azad) or Decitabine is an epigenetic drugs (16). In terms of molecular shape Azad is similar to deoxycytidine and usually used to demethylate of some gene's promoter, it is a powerful hypomethylating factor that combines into DNA and traps DNA methyltransferase in the form of a covalent protein–DNA adduct. And subsequently result in activated silent tumor-suppressor genes, growth-inhibitory, DNA repair and finally induce apoptosis (17, 18). Various scientific studies show that Azad, not only change the gene expression through demethylation of the gene's promoter, but it also can change gene expression independently from DNA demethylation. The effect of Azad on same gene expression in various environmental conditions can be different, and it can be the reason why patients treated with Azad show a variety responses (19). Different studies showed unusual DNA methylation and histone deacetylation is associated with tumorigenesis. Hence DNA methyltransferases (DNMTs) and histone deacetylases were identified as antitumor agents (20). Therefore, the purpose of our study was to distinguish whether Azad in addition to its inhibitory effects on DNA methyltransferases, can reduce HDAC3 and HDAC7 mRNA expression in NALM-6 (B cell precursor leukemia cell line), HL-60 (human promyelocytic leukemia cell line) cancer cell lines.

Methods

Chemicals:

The chemical such as Decitabine, RPMI 1640, Fetal bovine serum (FBS), Penicillin and streptomycin, Thiazolyl blue Tetrazolium Bromide (MTT), Eva green, DNA safe stain, RNA extraction kit, cDNA Synthesis Kits were purchase from Sigma Otsuka, America Pharmaceutical Inc., (Thermo scientific, MA, USA), (Gibco, Life technologies, Thermo fisher, USA), (Bio-idea, Iran), (Solis Biodyne, Estonia), (Biofact, Korea), (RNX-plus solution for total RNA isolation- sinaclon, Iran), (Thermo Fisher scientific, cDNA Inc. USA) respectively.

Cell culture:

Human cancer cell lines including NALM-6, HL-60 and normal cell (the Pasteur Institute, Tehran, Iran) were cultured in RPMI-1640 media enriched by 10% heat-inactivated fetal bovine serum (FBS), 50U/ml penicillin and 50µg/ml streptomycin at 37°C and 5% CO_2. We have obtained treatment does by the MTT assay and therefor NALM-6 and HL-60 cell lines were treated with 1µM Decitabine for 24 h (21).

Cell viability assay (MTT test):

Cells were cultured at a concentration of $1x10^5$ cells/well in 96-well micro plate and treated with AZad at different concentrations 0.1µM, 1µM and 5µM for 24, 48 and 72 h intervals. After treatment with AZad, 20µl of 5mg/ml MTT solution was added to each well and incubated for 4 h at 37°C. Next, 50µl of 20% acidified SDS added to the cells. Finally, absorbance of each well was measured at 570 nm by EPOCH Microplate Spectrophotometer (synergy HTX, BioTek, USA). Cell viability were expressed as a percentage in comparison to control. All tests were done 3 times (21, 22).

Quantitative polymerase chain reaction (qPCR):

Total RNA was isolated using an RNA extraction kit instruction protocol (RNX-plus solution for total RNA isolation, sinaclon, Iran). Similarly, cDNA was created using the transcriptor first strand cDNA synthesis kit (Thermo fisher scientific, cDNA Inc. USA). The expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (23). All experiments were repeated at least 3 times. The following primers for qPCR were used: HDAC3, forward 5'- CCA AGA CCG TGG CCT ATTT -3' and reverse 5'- AATGCAGGACCAGGCTATG -3'; HDAC7, forward 5'- GGACACCATGCAGATCATTCT -3' and reverse 5'- TGCACGTCC CAGTCTACAAT -3'; GAPDH, forward 5'- GAGCCACATCGCTCAGACAC -3' and reverse 5'- CATGTAGTTGAGGTCAATGAAGG -3'. Finally, all values were normalized to GAPDH expression levels.

Statistical analysis:

Statistical analysis was performed using two-tailed T-test. $P \leq 0.05$ was regarded as statistically significant. All statistical analyses were carried out by Graph Pad Prism 7.00 for windows (Graph Pad Software, San Diego, California, USA). Data were expressed as the mean ± (SD) of at least three independent experiments.

Results

In the present study, we have investigated HDAC3 and HDAC7 gene expression in HL-60, NALM-6 and normal cells. We also check out the effect of AZad on HDAC3 and HDAC7 mRNA expression in HL-60 and NALM-6 cells after and before treatment. (Fig.1- 5). Our results show that HDAC3 (Fig. 1A) and HDAC7 (Fig. 1B) mRNA expression in HL-60 cells increase significantly in comparison to normal cells. Whereas, HDAC3 (Figure 2A) and HDAC7 (Figure 2B) mRNA expression in HL-60 cells were declined after treatment with 1µM AZad. Similarly, HDAC3 (Figure 3A) and HDAC7 (Figure 3B) mRNA expression in NALM-6 cells elevated significantly when compared to normal cells. Nevertheless, HDAC3 (Figure 4A) and HDAC7 (Figure 4B) genes expression in NALM-6 cells was decreased after treatment with 1µM Azad.

Discussion

Scientific studies show that low concentration of AZad allows the intracellular cycles to remain active (21). In contrast, cellular toxicity associated with higher concentrations of AZad is undesirable, due to secondary effect related to apoptosis induced by the drug that would interfere with the changes in primary gene expression. Low dose of AZad induces hypomethylation, and this effect disappears at high doses (24).

Epigenetic histone alterations play a significant role in chromatin structure. Among enzymes controlling these processes, histone deacetylases (HDACs) can eliminate acetyl groups from histone tails, thus enhancing their interaction with DNA and leading to chromatin condensation (25). According to a Kara Gianni P study (2007), enhanced expression of HDAC3 in Acute myeloid leukemia (AML) is correlated with poor prognosis and response to treatment (26), and also in the study by Moreno DA et al. (2010), enhanced expression of HDAC3 has also been in childhood acute lymphoblastic leukemia (ALL) (27). Overexpression of HDAC 3 in several cancer types has been observed to be correlated with poor prognosis and response to treatment, including: prostate, breast, ovarian and colorectal cancers as well as T-cell acute lymphoid leukemia (T-ALL) (27, 28). Several studies has shown that overexpression of HDAC7 and its interaction with MEF2 family genes in ALL mostly inhibits transcription in lymphoid cells, causing failure of normal differentiation in lymphoid lineage (27, 29). Overexpression of HDAC7 is correlated with a weak prognosis in chronic lymphoid leukemia (CLL). It binds to c-Myc transcription factor gene, causing its overexpression and enhanced proliferation of cancer cells in CLL (30). Our findings show that Decitabine significantly reduce HDAC3 and HDAC7 mRNA expression in HL-60 and NALM-6 cancer cell lines. Specific HDAC inhibitors such as TSA (Trichostatin A) inhibits the Class I and II, but not Class III HDACs. TSA strongly inhibits HDAC1, HDAC2, HDAC3, HDAC4, HDAC6 and HDAC7 (13). While based on our results AZad reduce HDAC3 and HDAC7 mRNA expression. Therefore, it is suggested that the use of TSA and AZad at the same time may have synergistic effects and show better results, but it need more research. AZad is a powerful hypomethylating factor and Reducing HDAC3 and HDAC7 mRNA expression, we know the multiple therapy causes various side effects in patients. The use of multiple anticancer drugs is restricted due to toxicity in addition to the increase of drug resistance (31). Hence, new drugs are needed to ameliorate the clinical consequence and decrease the endurance to chemotherapy (32). Therefore, AZad can use as a candidate for hypomethylating and HDAC3 and HDAC7 reducing agents.

Conclusions

In summary, the results of the current study propose the possible involvement of HDAC3 and HDAC7 in leukemia's. Additional investigations are suggested to elucidate the potential role of HDAC3 and HDAC7 in carcinogenesis. These data showed that the effect of AZad are not limited to direct hypomethylation of DNMTs but also may indirectly affect other epigenetic factors, such as HDACs activity, through converging pathways.

Abbreviations

- HDACs: histone deacetylases
- AZad : 5-Aza-2'-deoxycytidine
- HMTs: Histone methyltransferases
- HDMs: Histone demethylases
- HATs: Histone acetyltransferase
- HDACs: Histone deacetylases
- HDACi : HDAC inhibitors
- ALL: acute lymphoblastic leukemia
- FBS: Fetal bovine serum
- MTT: Thiazolyl blue Tetrazolium Bromide
- qPCR: Quantitative polymerase chain reaction
- AML: Acute myeloid leukemia
- T-ALL: T-cell acute lymphoid leukemia
- CLL: chronic lymphoid leukemia
- TSA :Trichostatin A

Declarations

Ethics approval and consent to participate: This clinical protocol was approved by the Research Ethics Committee of the Shahid Sadoughi University of Medical Sciences (protocol number Ir.ssu.medicine.rec.1396.55). Signed informed consent was obtained from all individual participants included in the study. There are no human subjects or animals involved in the study.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this article.

Competing interests: the authors declare that they have no competing interests.

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Authors' contributions: Sina Dalvand and Sam Setayesh conceived and designed the study. Amin Namdari performed the article search and data extraction. Mohammad Hassan Meshkibaf evaluated the methodological quality of each study. Mohammad Farrokhifar and Mojtaba Haghi Karamallah

analyzed the data and wrote the paper, which was improved by Ashraf Alemi supervised the research. All authors read and approved the final manuscript.

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Figures

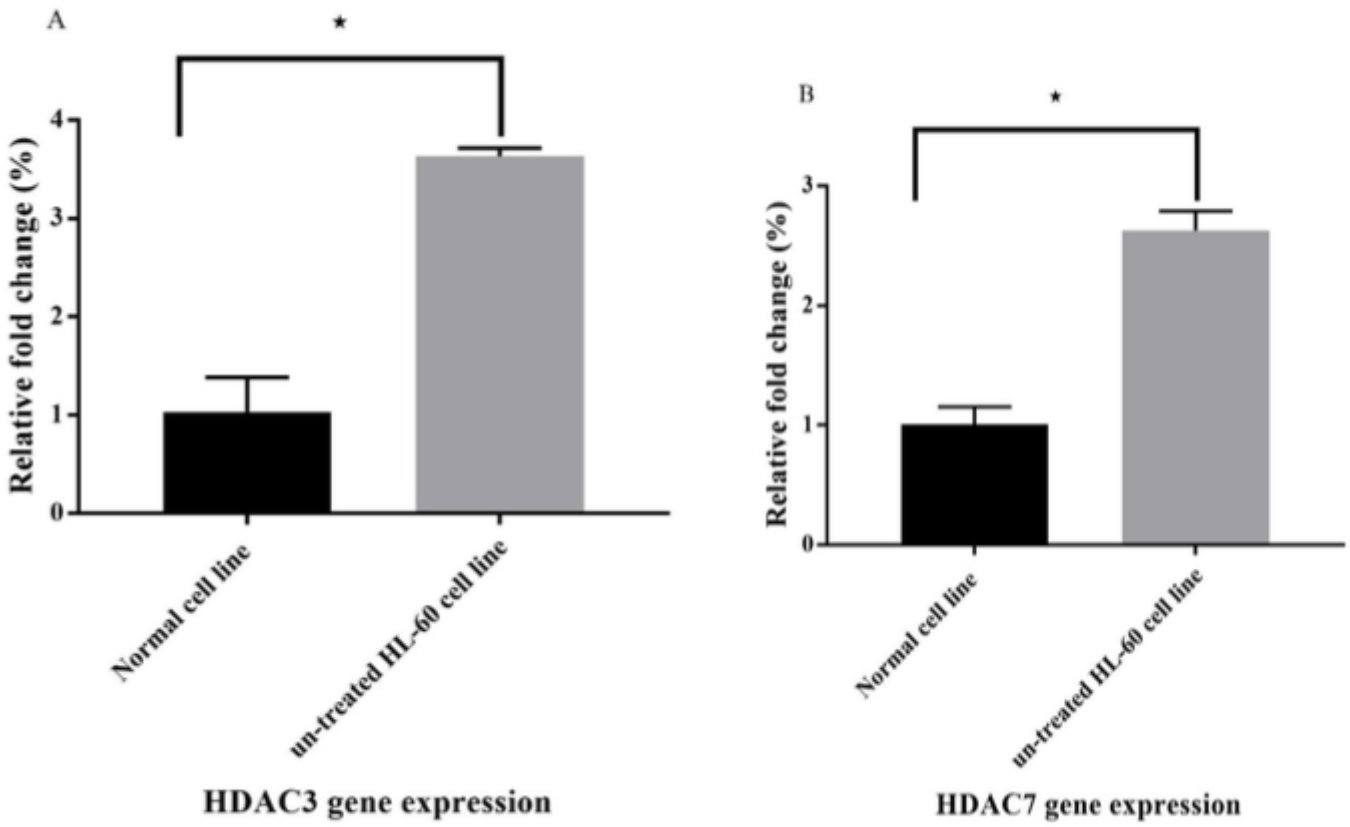

Figure 1

The Relative fold changes of HDAC3 and HDAC7 gene expression in un-treated HL-60 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. A) The Relative fold changes of HDAC3 gene expression, in un-treated HL-60 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. B) Relative fold changes of HDAC7 gene expression, in un-treated HL-60 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. Data were considered significantly with $p<0.05$. *$P<0.05$ vs. control cells.

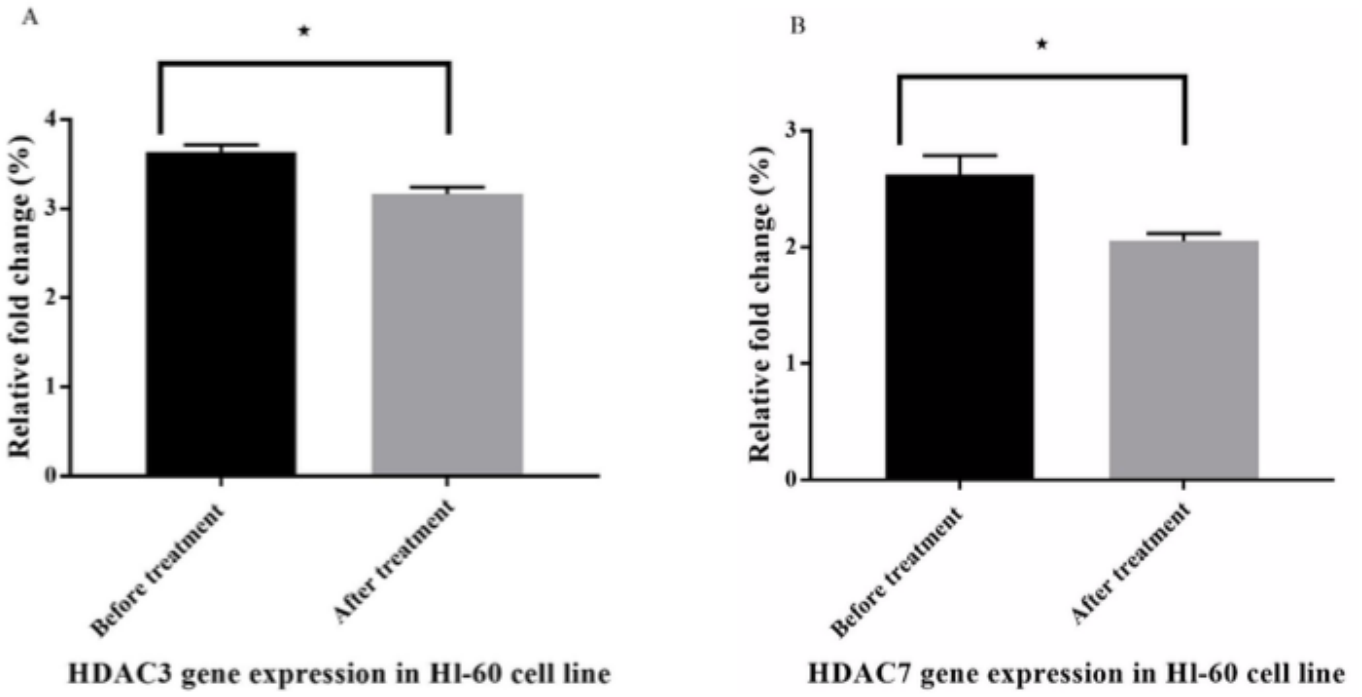

Figure 2

The Relative fold changes of HDAC3 and HDAC7 gene expression in treated (1μM AZad) HL-60 cell line and un-treated HL-60 cell line, after normalization to GAPDH. A) The Relative fold changes of HDAC3 gene expression, in treated (1μM AZad) HL-60 cell line and un-treated HL-60 cell line, after normalization to GAPDH. B) Relative fold changes of HDAC7 gene expression, in treated HL-60 cell line and the treated (1μM AZad) HL-60 cell line, after normalization to GAPDH. Data were considered significantly with $p<0.05$. *$P<0.05$ before treatment vs after treatment.

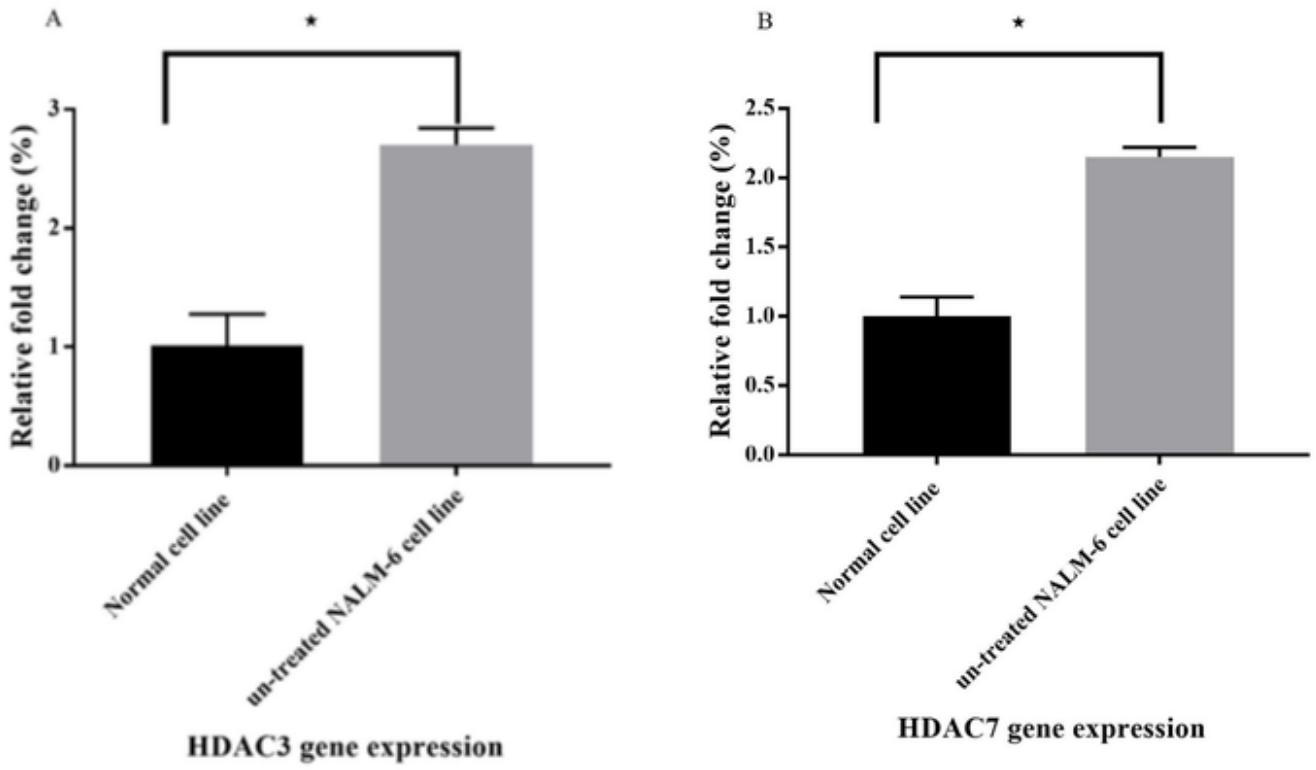

Figure 3

The Relative fold changes of HDAC3 and HDAC7 gene expression in un-treated NALM-6 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. A) The relative fold changes of HDAC3 gene expression, in un-treated NALM-6 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. B) The relative fold changes of HDAC7 gene expression, in un-treated NALM-6 cell line and in the healthy control group (as a calibrator), after normalization to GAPDH. Data were considered significantly with $p<0.05$. *$P<0.05$ vs. control cells.

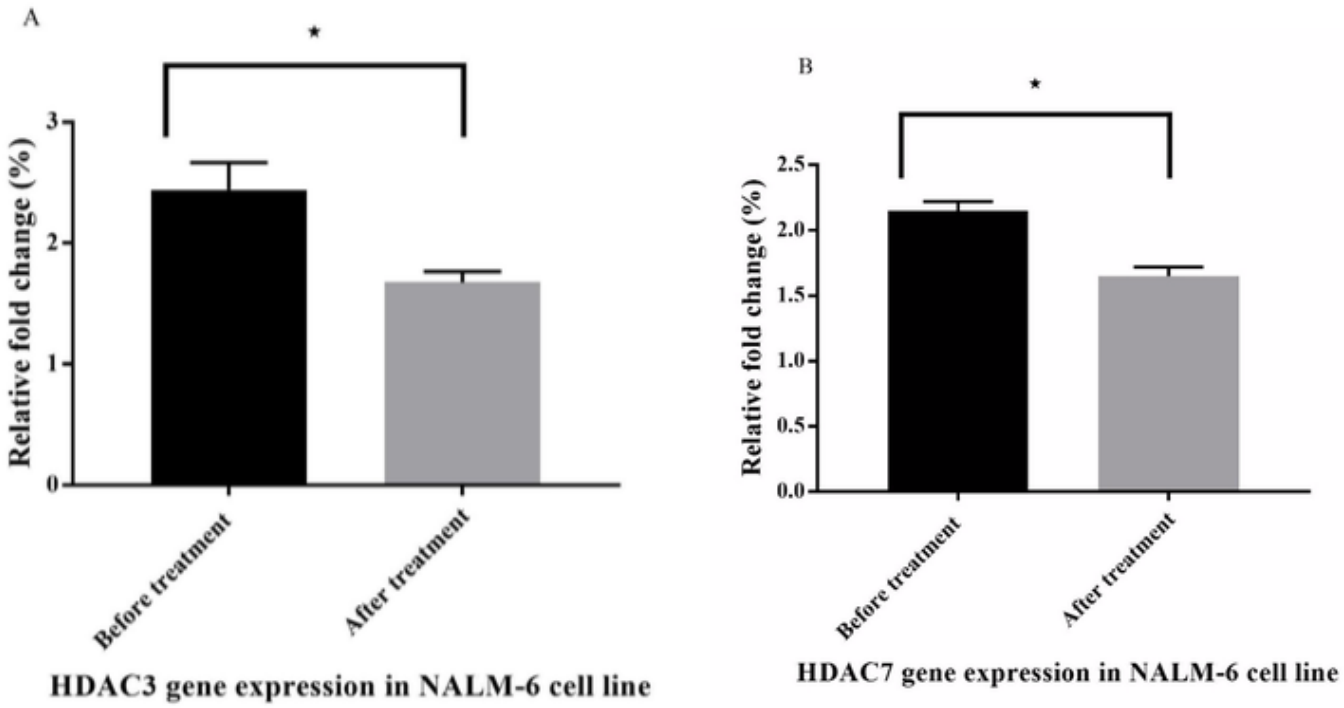

Figure 4

The Relative fold changes of HDAC3 and HDAC7 gene expression in in treated (1μM AZad) NALM-6 cell line and un-treated NALM-6 cell line, after normalization to GAPDH. A) The relative fold changes of HDAC3 gene expression, in treated (1μM AZad) NALM-6 cell line and the un-treated NALM-6 cell line, after normalization to GAPDH. B) The relative fold changes of HDAC7 gene expression, in treated (1μM AZad) NALM-6 cell line and the un-treated NALM-6 cell line, after normalization to GAPDH. Data were considered significantly with $p<0.05$. *$P<0.05$ before treatment vs after treatment.

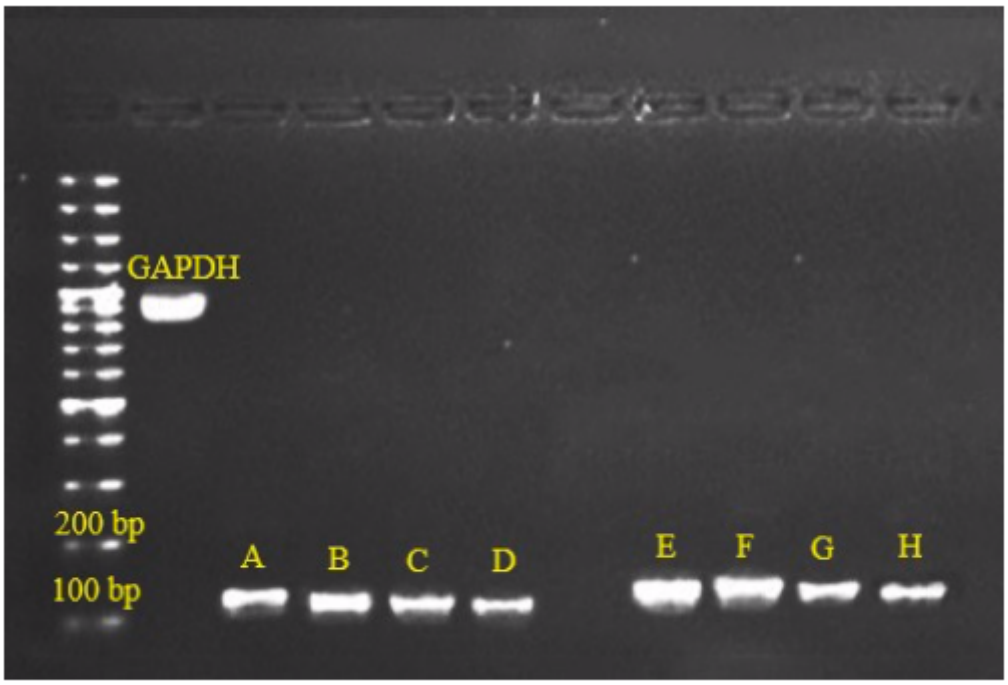

Figure 5

Real-Time PCR product. Represent Real-Time PCR gel patterns (qPCR) of HDAC3 and HDAC7, under non-saturating conditions on a 3% Agarose gel and stained with ethidium bromide. Fig. 5A) HDAC3 genes expression in un-treat HL-60 cell line (111 bp). Fig. 5B) HDAC3 genes expression in un-treat NALM-6 cell line (111 bp). Fig. 5C) HDAC3 genes expression in treated HL-60 cell line with 1μM AZad (111 bp). Fig. 5D) HDAC3 genes expression in treated NALM-6 cell line with 1μM AZad (111 bp). Fig. 5E) HDAC7 genes expression in un-treat HL-60 cell line (125 bp). Fig. 5F) HDAC7 genes expression in un-treat NALM-6 cell line (125 bp). Fig. 5G) HDAC7 genes expression in treated HL-60 cell line with 1μM AZad (125 bp). Fig. 5H) HDAC7 genes expression in treated NALM-6cell line with 1μM AZad (125 bp).